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Therapeutic Myocardial Angiogenesis and its Pharmacological Modulation

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To my parents whose inspiration is endless and
my family where my inspiration resides

Contents

ABSTRACT

Angiogenesis is the formation of new blood vessels induced by the proliferation and migration of endothelial cells. Because angiogenesis is a complex process, therapeutic efficacy may be improved by overexpression of combined angiogenic factors. Angiogenic effects of a single factor phVEGF-A (V) / phAng-1 (A) or their combination (V+A) were investigated in the myocardium. In the ischemic myocardium, phVEGF-A gene transfer alone increased myocardial capillary and arteriolar densities more than double and phAng-1 had a similar effect. Transient overexpression of VEGF-A boosts angiogenesis at the arteriolar level more than at the capillary level. Combination with Ang-1 further boosts this effect and induced up to a 7.5 fold increase in vessel growth. Overexpressed growth factors (V, A, and V+A) stimulated the mouse VEGF-Ang system whereas placebo plasmid had no such effects. Phosphorylation of antiapoptotic Akt and its downstream effector GSK3-α/β were lower during myocardial ischemia and there was no difference between any of the treatments. Upregulation of the endogenous ligands / receptors of the VEGF-angiopoietin system could be the mechanism for the therapeutic angiogenic booster effect during myocardial ischemia, a process that may occur independent of the Akt pathway. Hypercholesterolemia and aging are associated with impaired vascular density associated with downregulation of mVEGF, VEGFR-1, VEGFR-2, eNOS, Akt and upregulation of apoptotic protein p38 MAPK phosphorylation. phVEGF gene transfer in combination with simvastatin corrected impaired vascularity concurrently with stimulation of the VEGF system, increased eNOS production and reduced p38 MAPK phosphorylation. In the non-compromised heart enalapril and candesartan both specifically inhibit phVEGF-A induced myocardial angiogenesis associated with inhibition of hVEGF-A expression and decreased endogenous expression of the mVEGF ligand /receptor system. Combined overexpression of VEGF and angiopoietin has an angiogenic booster effect that occurs due to stimulation of the endogenous VEGF-Angiopoetin system. Aging and hypercholesterolemia have negative effects on angiogenesis. Commonly used cardiovascular drugs can stimulate or inhibit angiogenesis.

Key words: angiogenesis, arteriogenesis, ischemic heart, vascular endothelial growth factor, angiopoietin and receptor.

List of original papers

This thesis is based on the following papers:

I. Siddiqui AJ, Blomberg P, Wärdell E, Hellgren I, M Eskandarpour M, Islam KB, Sylvén C. Combination of angiopoietin-1 and vascular endothelial growth factor gene therapy enhances arteriogenesis in the ischemic myocardium. ***Biochemical Biophysical Research Communication.*** 2003; 310: 1002-1009.

II. Siddiqui AJ, Gustafsson T, Widegren U, Grinnemo KH, Dellgren G,Hao X, Mansson-Broberg A, Fischer H, Sylvén C. Therapeutic endogenous ligand/receptor induction of the VEGF-angiopoietin system after intramyocardial gene transfer during myocardial ischemia. ***Journal of Molecular Medicine***. (*Under revision).*

III. Siddiqui AJ, Gustafsson T, Fischer H, U Widegren U, X Hao, Mansson-Broberg A, Grinnemo KH, Dellgren G, Sylvén C. Simvastatin enhances myocardial angiogenesis induced by vascular endothelial growth factor gene transfer. ***Journal of Molecular and Cellular Cardiology.*** 2004; 37: 1235-1244.

IV. Siddiqui AJ, Mansson-Broberg A, Gustafsson T, Grinnemo KH, Dellgren G, X Hao, Fischer H, Sylvén C. Antagonism of the renin angiotensin system counteracts cardiac angiogenic VEGF gene therapy. ***American Journal of Hypertension***. 2005; 18:1347-1352.

Abbreviations

IHD- Ischemic Heart Disease

EC- Endothelial Cell

VEGF- Vascular Endothelial Growth Factor

VEGFR-Vascular Endothelial Growth Factor Receptor (1/2)

sVEGFR-1- Soluble Vascular Endothelial Growth Factor Receptor-1

RTK- Receptor Tyrosine Kinase

Ang- Angiopoietin (1/2)

CMV- Cytomegalovirus

CABG- Coronary Artery Bypass Graft

PTCA- Percutaneous Transluminal Coronary Angioplasty

$ApoE^{-/-}$- Apolipoprotein-E Gene Knockout

HFD- High Fat Diet

MAPK- Mitogen Activated Protein Kinase

Akt /PKB- Protein Kinase B

ENOS- Endothelial Nitric Oxide Syntathase

GSK3-α / β - Glycogen Synthetase Kinase (α / β)

RAS- Renin Angiotensin System

ACE- Angiotensin Converting Enzyme

INTRODUCTION

General Background

Treatment of IHD remains as major challenge worldwide. Early revascularization limits ischemic injury of the myocardium, which may prevent complications of ischemia such as death of myocardial tissue. A large number of patients do not meet criteria for CABG or PTCA whereas others have residual symptoms of ischemia despite such interventions. IHD, however remains one of the leading causes of the total morbidity and mortality. Although ischemic injury or tissue hypoxia are important stimulators for angiogenesis, downregulation of endogenous drive or alteration of angiogenic related molecules and their receptors during ischemia could be considered as mechanisms for insufficient collateral and vascular development. Importantly, studies suggest that tissue hypoxia is not always efficient to induce an angiogenic response (1). Furthermore, an imbalance of angiogenic factors and dysfunction of the receptor signal pathways should not be excluded (2).

Angiogenesis (the growth of new blood vessels) is a tightly regulated process in adult tissues that occurs as endothelial cells from pre-existing vessels proliferate and migrate. Post-natal angiogenesis can occur due to physiological demand (e.g., wound healing and menstrual cycle) or to pathological events (e.g., tumor, diabetic retinopathy, arthritis etc.). Endothelial cells are the key players in the angiogenic process. A number of defined

angiogenic molecules or growth factors have been identified as important mediators for this complex multistage process.

Angiogenic factors and their receptors:

Angiogenesis is orchestrated by a wide variety of molecules and cytokines that are activated during several physiological and pathological conditions. Principal mediators include VEGF and its family, fibroblast growth factors (FGF), angiopoietins, platelet derived growth factors (PDGF), hepatocyte growth factor (HGF) and many more (3).

This thesis is based on studies of the VEGF and angiopoetin system.

Vascular endothelial growth factor-A (VEGF-A), one of the key angiogenic factors, has been explored in several clinical studies (3, 4). The VEGF family consists of six members: VEGF-A (VEGF-1), VEGF-B (VEGF-3), VEGF-C (VEGF-2), VEGF-D, VEGF -E and Placental growth factor (PLGF). Although angiogenesis is a complex process with the involvement of many signaling pathways, the VEGF (VEGF-A)-signaling is a critical-rate-limiting step in the process (5). VEGF-A has five important splice variants or isoforms: VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206} depending on the number of amino acids. All the VEGF-A isoforms have a secretory signal sequence while VEGF-A_{165} is the predominant isoform and a diffusible protein but it may remains bound to cell surface and extra cellular membrane (6). The biological actions of VEGF are mainly mediated by two structurally related receptor tyrosine kinases: VEGFR-1/Flt-1 and VEGFR-2 / Flk-1. Although VEGFR-2 is

essential for many of the VEGF mediated actions, studies have shown that VEGFR-1 knocked out mice suffer from fetal death due to vascular defects (7). Both VEGFR-1 and VEGFR-2 are membrane bound vascular endothelial cell specific receptors whereas the third receptor (VEGFR-3) is expressed mainly in the lymphatic endothelial tissue. Because Neuropilin (NRP-1/2) has been identified but have short intracellular domains it is unlikely to act as an independent receptor for VEGF (8). It may act as a co-receptor for VEGFR-2 (9). NRP-1 deletion is also reported as embryonic lethal due to vascular cardiac malformation (10, 11) but its direct role in the postnatal angiogenesis is unclear.

Angiopoietins (Ang-1/2) are important co-players in vascular development, remodeling and maintenance and mediates angiogenic functions through activation of the Tie-2 receptor located at the endothelial cells. Like VEGF, the angiopoietins are also secreted proteins and Ang-1 acts locally whereas Ang-2 freely diffuses in the tissues (12). Both Ang-1 and Ang-2 knockout are embryonically lethal due to cardiac defects and vascular abnormalities, suggesting that the molecules are essential for vascular development (13). In ischemic hearts Ang-2 is reported to be upregulated (14) whereas Ang-1 is downregulated (15). Importantly, Ang-1 is associated with endothelial survival, angiogenesis and recruitment of endothelial progenitor cells (16-19). These data suggest that Ang-1 may also have cardioprotective function, making it a possible therapeutic tool. In contrast, Ang-2 acts as natural antagonist to Ang-1, inhibiting phosphorylation of Tie-2. These signalling cascades may lead to vessel destabilization and Ang-2 may further progress the angiogenic process in the presence of VEGF (20). Recently, Ang-3 and Ang-4 have been identified. They work complementary to each other and have binding affinity

to Tie-2 (21). Tie-1 is another receptor tyrosine kinase like Tie-2 but it may participate in fluid exchange control across capillary ends and in hemodynamic stress resistance (22, 23).

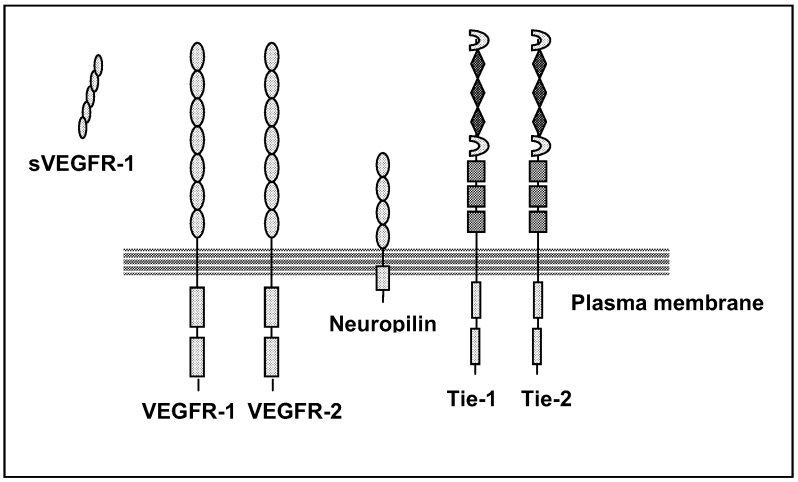


Figure 1: VEGF and angiopoetin receptor system.

Receptor Tyrosine Kinase

Receptor tyrosine kinase is a group of transmembrane polypeptides containing a protein kinase domain in their intracellular region, a transmembrane and extracellular domain. Both VEGF (VEGFR1/2) and angiopoetin (Tie-1/2) receptors belong to this family (Fig.1) and thus share much similarity in their signal transduction. The extracellular portion of the receptor is responsible for binding to a specific growth factor or signal molecule and thus responsible for signal transduction. Receptor mediated signal transduction is a rapid three stage process (Fig.2) initiated by the signal reception at the plasma membrane followed by transduction, a process that usually has several sub-steps followed by a cellular response. Signal transduction from receptor tyrosine kinase

initiates several cellular events including proliferation, migration, cellular shape change, production of matrix metalloproteinase's (MMPs) and initiation of transcription factors.

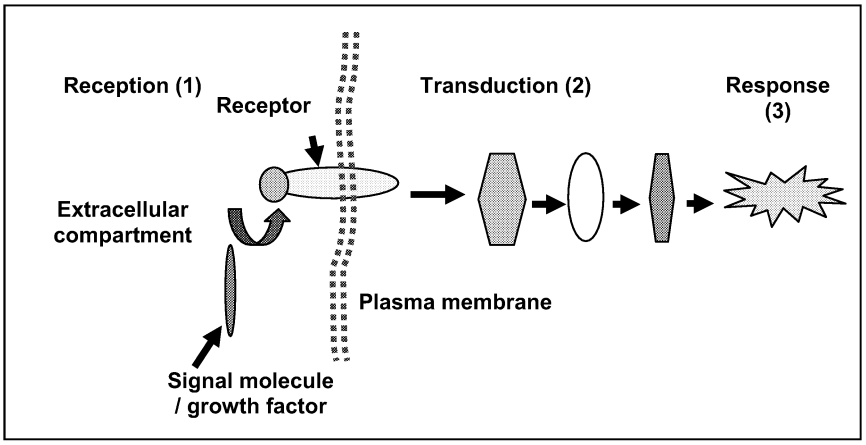


Figure 2: Receptor mediated signal transduction can be simplified as a three step process: (1) Reception, (2) Transduction and (3) Response.

Molecular Basis of Vascular Growth

Development of the vascular network is mediated by three distinct processes or mechanisms: vasculogenesis, angiogenesis and arteriogenesis (24). Vasculogenesis is an in situ formation of primitive blood vessels from angioblast or progenitor cells mainly attributed to an embryonic process, but recently it has also been observed in adult tissue (25). This primitive vascular plexus later passes through extensive remodeling and gives rise to a mature vascular network. By contrast, angiogenesis is a postnatal neo-vascularization event that occurs in different physiological and pathological conditions mediated by growth factors and cytokines. The angiogenic process has been characterized as complex and multistage with initial dilation (by nitric oxide) of the vessel wall due to activation of endothelial cells by angiogenic growth factors. Activated endothelial cells

secrete MMPs that degrade the basement membrane. Thereby the endothelial cells can escape from the residing vessels.

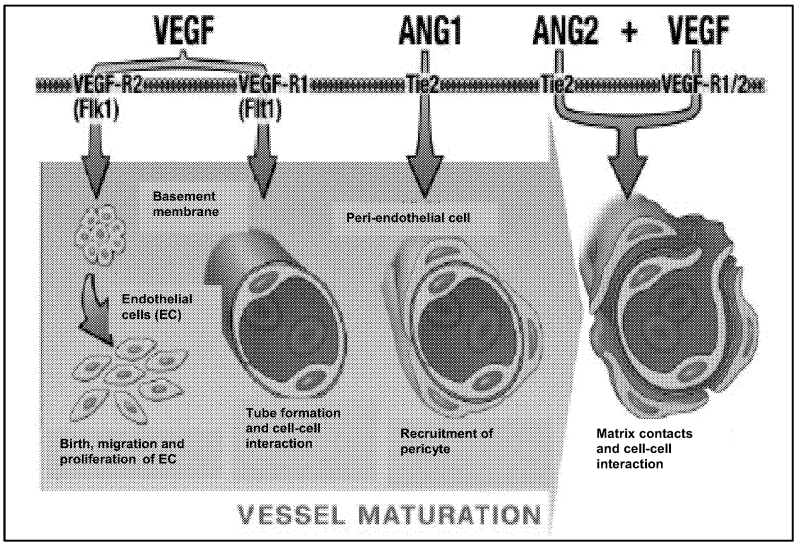


Figure 3: Angiogenic vessel transforms and matures to achieve functional capacity meeting local tissue demands. Ang-1 is an important factor that stimulates recruitment of pericytes or smooth muscle cells and thereby provides vessel stability in concert with VEGF (adapted from ref. 7).

Then the EC migrate, proliferate, and organize to form a luminar structure and connect to the local vascular network or form a new network of blood vessels (Fig.3). Finally the endothelial cell re-differentiates to adapt a quiescent phenotype. These blood vessels often named nascent blood vessels are capillary-like structures. Further remodeling of newly formed capillaries is important for long-term survival, functionality and meeting of local tissue demands. Indeed the newly formed capillaries can disappear or regress when supporting cells such as smooth muscle cells or pericytes are absent and the stimulating growth factor is withdrawn (26). Ang-1 / Tie-2, PDGF-BB / PDGF-β, transforming

growth factor (TGF)- β, Sphingosine-1-Phosphate-1 (S1P1) / endothelial differentiation sphingolipid G-protein -coupled receptor-1 (EDG1) , and probably ephrin-B2 / Eph B4 are important molecules that have been identified in relation to post-natal vascular remodeling and stabilization by the recruitment of supporting cells (27, 28). Local tissue demands and hemodynamics are critical determinants for vessel remodeling and stabilization.

Arteriogenesis uses pre-existing conduit vessels in the ischemic region in response to occlusion of a large vessel. The main driving force for arteriogenesis is increased shear stress due to higher nutritive flow in the corresponding vascular bed (29).

Myocardial Gene Therapy:

Overexpression of growth factors in an ischemic heart has been explored with viral or plasmid vectors or with protein formulation to transfer the angiogenic agent of interest to induce therapeutic angiogenesis (3, 30, 31). Uptake of viral vectors into the target cell has been suggested to occur via a specific receptor mediated mechanism. A long-term expression of induced growth factor can be achieved via viral vectors while inflammation, immunogenic and cost of production are important disadvantages (32, 33). Protein formulation may be a choice due to the precise action and control of the desired dose but short half-life, systemic spread and high cost are barriers for therapeutic angiogenesis (34). In contrast plasmids are easy to produce and purified although expression of the reporter gene is transient. Plasmids are small circular extrachromosomal DNA multiplied in prokaryotic cells (*Escherichia coli*) and can carry the desired gene of interest in gene therapy (35). The exact mechanism for plasmid

transportation is unknown but it has been suggested that plasmids enter a host cell by endocytosis (Fig.4), a simple transport mechanism across the cell membrane. However, much of injected plasmid is degraded or destroyed at the extracellular level. Upon entering the host cell plasmid is transported into the nucleus where it remains essentially as extrachromosomal. Plasmid derived expressed gene stimulates specific receptor at cell membrane or it may initiate intracellular mechanism depending on type of protein and cell that has been transfected.

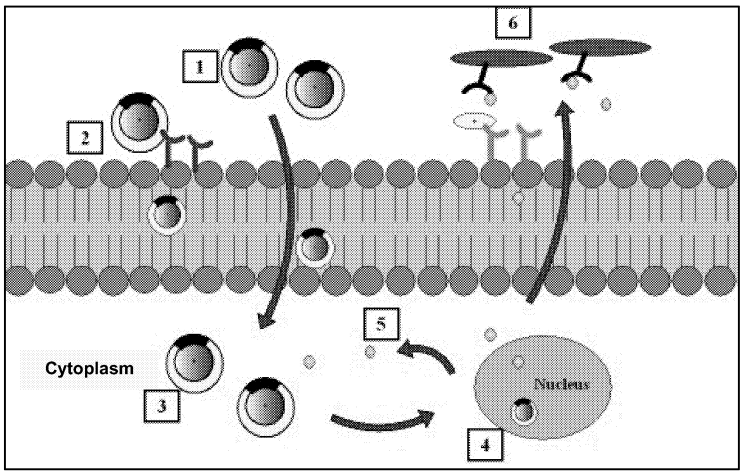


Figure 4: Plasmid cellular uptake is considered to occur via endocytosis (1), toll like receptor may play a role (2), upon entering in cell cytoplasm (3) plasmid is vulnerable to endonucleases. From the cell cytoplasm plasmid is transported to the nucleus (4) and preferentially is extrachromosomal. Expressed protein can initiate intracellular (5) or extracellular (6) events depending on type of protein and transfected cell.

Therapeutic Myocardial Angiogenesis as a Basis of Combination Therapy

Therapeutic myocardial angiogenesis may be used to treat ischemic heart disease by delivering angiogenic gene to the ischemic myocardium. The treatment is aimed to improve myocardial function due to enhancement of regional blood flow from neo-angiogenesis. This may also limit further damage to the heart from the ischemic injury. Clinical trials with VEGF or FGF gene transfer seem to be safe and early improvement of symptoms has been reported (36-39). Although investigations are under way, until now myocardial angiogenic gene therapy has not unequivocally been demonstrated to have clinical efficacy (40). Study design, inadequate angiogenic therapeutic gene expression as well as low efficacy might be causes. A combination of angiogenic growth factors has been proposed to enhance the angiogenic response (41- 44). Because angiogenesis is a complex process, it is hypothesized that therapeutic efficacy may be improved with overexpression of combined factors.

Advanced age and increased cholesterol levels are important factors in the development of ischemic heart disease. Aging is also associated with impaired angiogenic capacity, which may suggest a potential link or interaction of aging with the angiogenic molecules (45). Interestingly it has been recognized that some of the common used cardiovascular drugs tend to interfere with the angiogenic process (46). Clinical studies have shown that statins prevent both primary and secondary coronary artery disease (47- 49). The mechanisms behind these observed beneficial clinical effects have been mainly attributed to changes in blood lipids, such as reduced cholesterol and triglyceride levels. However,

there are statin-induced cardioprotective, anti-inflammatory as well as proangiogenic mechanisms (50-53) and these actions are considered to be unrelated to changes in blood lipid profiles. RAS inhibition by ACE inhibitor (ACEi) and angiotensin II antagonists are drugs commonly used in ischemia related heart diseases and heart failure. A significant reduction of the mortality and improvement of quality of life have been achieved by RAS inhibition. However, cardiac RAS is also associated with varieties of cellular responses such as inflammation, angiogenesis and apoptosis (54).

AIMS OF THE STUDY

1. To compare the angiogenic effects of overexpression of a single factor VEGF-A, Ang-1 or their combination in the ischemic and normal myocardium.
2. To characterize the mechanism of the therapeutic angiogenic effect of VEGF-angiopoietin overexpression on the basis of adaptations of the endogenous VEGF-angiopoietin system.
3. To investigate the angiogenic effects of VEGF-A gene transfer in hypercholesterolemia and aging and the effects of simvastatin treatment on myocardial angiogenesis.
4. To investigate the effect of inhibition of the renin angiotensin system (RAS) on myocardial angiogenesis induced by VEGF-A gene overexpression.

MATERIALS AND METHODS

Animals:

Male C57Bl/6 mice, 25-30 gm and 6-8 weeks old (B&K, Sollentuna, Sweden) were used in the study. (**Paper I-II, IV)**

Male ApoE$^{-/-}$and C57Bl/6 mice were used for the experiments. The mice were obtained from (B&K, Sollentuna, Sweden) and the ApoE$^{-/-}$were fed with high fat diet (HFD) (Lactamin, Sweden) starting at their 6-8 weeks of age to 12 months to induce and accelerate the hypercholesterolemia. (**Paper III)**

Plasmids:

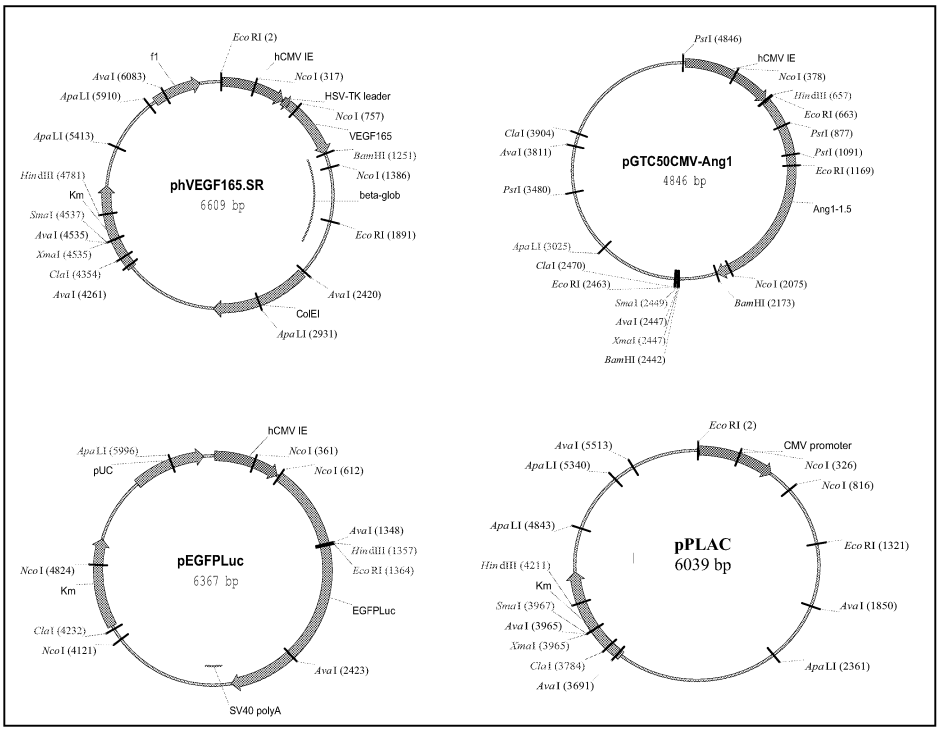


Figure 5: Plasmids used in the experiments

phVEGF-A_{165}: It is an eukaryotic expression vector encoding for the 165 amino acid isoform of human VEGF, driven by a human cytomegalovirus immediate early (HCMV IE) promoter/enhancer.

phAng-1: Contains the expression of the human angiopoietin-1 cDNA, driven by the human CMV promoter and generated by PCR from cDNA prepared from a HUVEC-C cell line (ATCC 1730-CRL).

pEGFP-Luc: Plasmid driven by CMV virus and upon trasnfection expresses both green fluorescent protein (GFP) and luciferase.

Placebo Plasmid: Has a similar construction as phVEGF-A_{165} but contains no transgene.

For the propagation of plasmids *E. coli* DH5α from Life Technologies (Gaithersburg, MD, USA) was used as host. Plasmids were purified using Qiagen kits (Hilden, Germany) according to manufacturer's instructions. Purity was checked by gel electrophoresis and by optical density at 260 nm and 280 nm.

Experimental Designs

The studies were approved by the local Animals Ethics Committee.

Paper I-II

The mouse was anesthetized with a 100 µl injection of a combination of midazolam (5 mg/kg) and medetomidin (0.5 mg/kg) diluted with normal saline and then intubated and adapted to a ventilator (Zoovent, UK). A left thoracotomy was done**.**

Nonischemic Model: The heart was visualized by retracting the pericardial sac. The left ventricular wall was targeted for direct intramyocardial injection (Fig. 6) of phVEGF-

A_{165} (5 µg in 10 µl saline), phAng-1 (5 µg in 10 µl saline) or a combination of phVEGF-A_{165} + phAng-1 (5+5 µg in 10 µl saline). Control mice received 10 µl of saline injection.

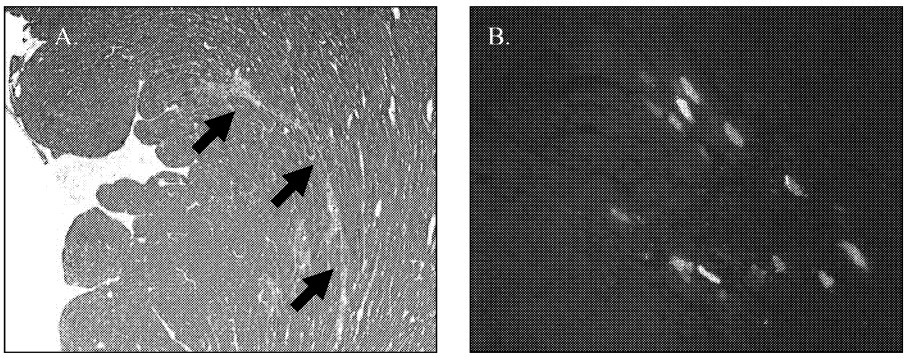


Figure 6: Intramyocardial gene transfer. (A) Injection scars (arrows) at left mid intra-ventricular area after intramyocardial injection (haematoxylin-eosin stain). (B) GFP transfected cardiomyocytes (40 x magnifications).

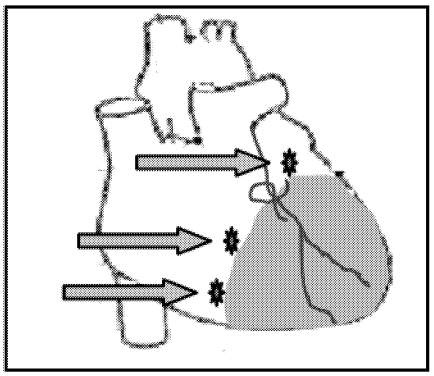


Figure 7: Ligation of LAD coronary artery and injection of plasmid into periinfarct area.

Acute myocardial infarction (AMI) model: Following the above protocol, the left anterior descending artery (LAD) was identified by retraction of the pericardium. The distal one-third of LAD was ligated with a 7.0-proline suture. In the sham operation, the same protocol was followed with no ligation of the artery. Myocardial infarction was confirmed by color change and dyskinesia of the lower anterior lateral wall, including the apical region of the left ventricle (Fig.7). phVEGF-A_{165} (5 µg in 10 µl saline), phAng-1

(5 µg in 10 µl saline) or a combination of phVEGF-A_{165} + phAng-1 (5+5 µg in 10 µl saline) was injected at 2 or 3 points around the infarcted zone in the left ventricular wall with an insulin syringe. The control mice received saline (10µl) (Paper I) or placebo plasmid (5 µg in 10 µl saline) (Paper II) as above protocol. Mice were sacrificed and hearts were harvested up to 14 (D) days after AMI.

Paper III

The $ApoE^{-/-}$(1 year) and C57Bl/6 (6-8 weeks, young) mice were randomly divided into three groups-saline, phVEGF-A, phVEGF-A+ Statin (n=5 in each), respectively. phVEGF-A_{165} or placebo plasmid (5 µg in10 µl saline, in both cases) was injected into the left ventricular wall after a thoracotomy. Mice received daily simvastatin treatment orally (70mg/kg) as described previously (55) for 10 consecutive days from the first day of the gene transfer.

To investigate specific effects of simvastatin on the VEGF system, mouse mVEGF expression at protein level (24 hours and 10 days) and VEGF, Flt-1/VEGFR1 and FlK-1/VEGFR2 expression at mRNA level with or without simvastatin treatment (10 days) were investigated in subsets (n=5 in each) of $ApoE^{-/-}$ mice hearts.

Simvastatin (Zocord, Merck): Collected from local hospital pharmacy (Apotek) and pulverized and activated with 2% methylcellulose (55).

Paper IV

Mice were given a single intramyocardial injection of phVEGF-A_{165} (similar protocol of Paper I). Enalapril or candesartan were given subcutaneously for 10 consecutive days.

Hearts were harvested and capillary count done by immunohistochemistry. With similar design, groups of mice were sacrificed after 24 hours for the determination of tissue expression of phVEGF-A protein, mRNA expression of mouse VEGF-A, and VEGF receptors 1 and 2 and after pEGFP-Luc transfection for luciferase expression.

hVEGF and hAng-1 and mVEGF Protein Expression

hVEGF-A protein was determined by ELISA following VEGF- A gene transfer in the myocardium at three different points: 24, 48, and 96 hours after injection.

Immediately after euthanasia hearts were taken out individually and cut into small pieces and kept in liquid nitrogen. Excess blood was rinsed off from heart tissue with 20 mM Hepes, pH 7.4 buffer. Thereafter each heart tissue was taken into an Eppendorf tube with 400 µl homogenization buffer (20 mM Hepes pH 7.4, 1.5 mM EDTA, 0.5 mM PMSF, 0.5mM benzamidine, and 10 µg/ml trypsin inhibitor) and minced with a homogenization knife during 1 minute on ice. Each homogenized substance was taken to another Eppendorf tube and centrifuged for 10 min at 14000 x rpm (4 ^{0}C). Finally the supernatant was collected in two new Eppendorf tubes at -70^{o} C. Later all frozen samples were analyzed at the same assay for hVEGF-A protein with standard human VEGF immunoassay detection kit (Quantkine, R&D system) according to the instruction.

After 24-48 hours of phAng-1 injection, hAng-1 expression was determined by immunohistochemistry on paraffin sections.

mVEGF immunodetection were also done according to the manufacturer's instruction (Quantikine, R&D system).

IMMUNOHISTOCHEMISTRY

Capillary Analysis

At sacrifice hearts were immediately collected and the ventricle was dissected into two parts embedded in Oct compound (Histolab, Sweden) and frozen in liquid nitrogen, then kept in -70^{0}C until sectioned. For capillary morphology and immunochemistry 5μm sections of ventricular tissue in super frost slides were incubated with Griffonia Bandeiraea Simplicifolia Isolectin B4 (GSL-I-B4, Vector Laboratories) followed by a second incubation with ABComplex. Finally capillaries were visualized using DAB with supplementation of 0.03% hydrogen peroxide. Capillaries were counted using a LCD camera (Olympus, Japan) connected with a microscope at 40x magnification. Approximately 12-16 photos around the injection site from adjacent 3-5 sections were used for counting capillaries in the ventricle. The capillary count was analyzed with an image analysis program according to pre-set criteria as regards to the size and developed color (Micro Image, Olympus). The quality of computer analysis had previously been checked against manual counting.

Arteriolar Analysis

Ventricular sections were stained for immunohistochemical analysis with α-actin, a smooth muscle specific antibody to analyze and count arterioles. The α−actin containing vessels were defined as arterioles although a more narrow definition is that arterioles should be ≥ 10 μm in diameter (56). Consequently the density of α−actin stained vessels was analyzed according to size: small and large arterioles (≥ 20 μm diameters and round

shape). Briefly, frozen sections of ventricle were fixed in 2% formaldehyde diluted in 1x PBS for 10 minutes then incubated with normal rabbit serum as a blocking solution for 30 minutes. Sections were incubated with primary antibody α-actin (Sigma) overnight in 1:250 dilutions. Finally rabbit anti-mouse secondary antibody (FITC, Dako) was used to visualize the blood vessels. Blood vessels found stained with the antibody around the injection site in normal heart and entire section in AMI group was counted and analyzed on 20x magnification in a fluorescence microscope by an individual who was blinded in this study.

Endothelial Progenitor Cells (EPC)

Serial sections of ventricular tissue (5-μm) were prepared for rat anti mouse $CD34^+$ (Serotec) and VEGFR-2 / Flk-1 (R&D system) immunohistochemistry. Endothelial progenitor cells (EPC) in myocardium were identified when both $CD34^+$ and VEGFR-2 were co-expressed (57). FITC-conjugated rabbit anti-rat and biotinylated rabbit anti-mouse (DAKO) was used as secondary antibody against $CD34^+$ and VEGFR-2, respectively, and counter-stained with DAPI.

eNOS Immunohistochemistry

5-μm ventricular sections were incubated with primary antibody (Sigma) at 1:250 dilutions overnight at 4^0 C followed by biotinylated secondary antibody (Dako) incubation. Sections were visualized with diaminobenzidine and counterstained with hematoxyline. The eNOS expression in the ventricular section was analyzed semi-quantitatively as described before (58). The eNOS immunohistochemistry stained

capillary endothelial cell, cardiomyocyte, small blood vessels and the large vessels of heart and scored on a 0 to 10 scale. A number of positive signals (brown cells) were counted and mean values were determined from the total score of three consecutive sections from each heart.

RNA ISOLATION AND cDNA PREPARATION (Paper II-IV)

RNA was isolated with Trizol reagent (Life technologies, USA) as described by the manufacturer. Total RNA was assayed by spectrophotometer (A260nm/A280nm) to assess purity and concentration. RNA sample was also checked in 1-% agarose gel containing ethidium bromide. The 5s, 18s and 28s rRNA bands were visualized under UV light (BioRad, USA). RNA samples were diluted with water and stored at -70^0 C. 2 µg total RNA from each sample was reverse transcribed to prepare cDNA by Superscript RNase H reverse transcriptase (Superscript II, Invitrogen, USA /GIBCO BRL) with the random hexamer primers according to the manufacturer's specification.

mRNA Quantification

Real-time PCR was used for measurement of specific mRNAs (ABI-PRISMA 7700 Sequence Detector, Perkin-Elmer Applied Bio systems Inc., Foster City, CA, and USA). Oligonucleotide primers and TaqMan probes were designed by using Primer Express version 1.5 (Perkin-Elmer Applied Biosystems Inc.). The probes were designed to cover exon-exon boundaries to avoid genomic DNA amplification. Because endogenous control to correct for potential variation in RNA loading and quantification, 18S were used. The oligonucleotide sequences for the primer pairs and probes are shown in Table 1. and

hVEGF-A: 5´TGTGAATGCAGACCAAAGAAAGA, 3´TGCTTTCTCCGCTCTGAGC
PROBE: FAM-AGGCCCACAGGGAACGCTCCAG-TAMRA
hAng1: (Primer + probe) Assay on demand (ID: 00919197), Applied Biosytems, USA.
VEGF: 5´ CCACGTCAGAGAGAGCAACATCA, 3´ TCATTCTCTCAGGCGGCTTTG
Probe: FAM-CAGGGAAATAAACGGA-TAMRA
VEGFR-1: 5´ TGTGAACGGCTGCCCTATGATG, 3´ GCCAAATGCAGAGGCTTGAA
Probe: FAM-CCCAGGATTGGAACC-TAMRA
VEGFR-2: 5´ TCTGGCTCCTTCTTGTCATTGTC, 3´GCTCATCCAAGGGCAATTCAT
Probe: FAM-ACGGCATCCAGGA-TAMRA,
Angiopoietin-1: 5´ GTATGTGCAAATGCGCTCT, 3´ATGATTTTGTCCCGCAGTGTAGA
Probe: FAM- CGATGCCTGTGGCCCTTCCAATCTA-TAMRA
Angiopoietin-2: 5´ CTGCAAGTGTTCCCAGATGCT, 3´ GTGGGTAGTACTGTCCATTCAAGTT,
Probe: FAM- TGGAAGCCCAAGTATTAAATCAGACCACGAGA-TAMRA
Tie-2: 5´CAGTGATGTCTGGTCCTATGGTGTA, 3´GCACGTCATGCCGCAGTA
Probe: FAM. TGCCTCCTAAGCTAACAATCTCCCAGAGCAZ-TAMRA

Table 1: The primers and probe sequences used in the experiments.

hAng-1 was obtained as assay on demand (Hs 00919197_m1, applied Bio systems Inc.). 18 s was supplied as a TaqMan® Reagent kit from Applied Biosystems with TAMRA quencher and used according to the manufacturer's instruction. All reactions were performed in 96-well MicroAmp Optical plates. Amplification mixes (25µl) contained diluted sample cDNA (5µl), 2X TaqMan Universal PCR Mastermix, forward and reverse primers and probe for the specific mRNAs, as well as 18S mix. Thermal cycling conditions included 2 minutes at 50° C and 10 minutes at 95° C before the onset of the PCR cycles, which consisted of 40 cycles at 95° C for 15 seconds and 65° C for 1 minute. The fluorescence threshold was set in the exponential phase of product formation (Fig.8). Control experiments revealed approximately equal efficiencies over different starting

template concentrations for target genes and 18S. Target gene and 18S were amplified in a single-plex experiment in duplicate and repeated for three times.

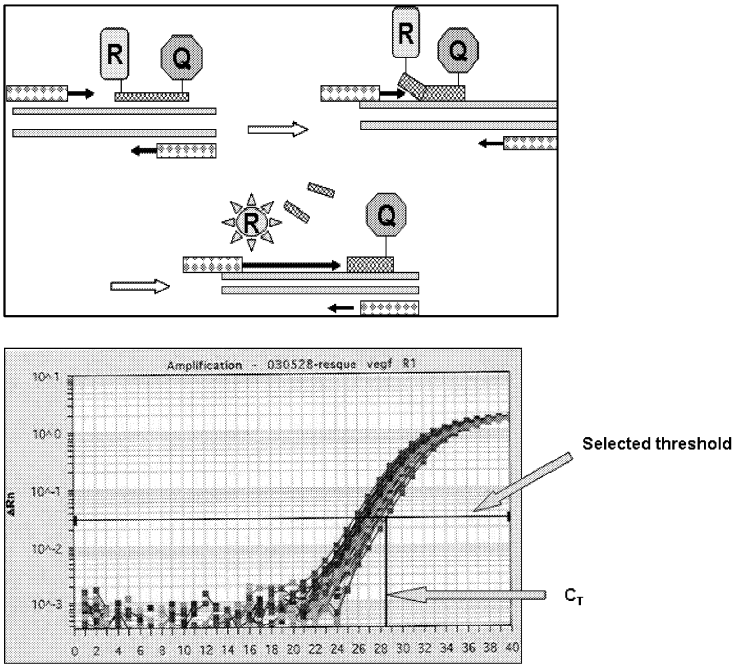


Figure 8: Real time PCR Taqman. The amount of PCR product is quantified in real time by using special probes and reporter dye (R) that fluorescence when the quencher (Q) is removed from the mRNA fragment during the PCR extension cycle. The number of PCR cycles (termed CT value) needed to reach a given fluorescence signals (threshold) is related to the amount of mRNA in the sample.

Dilution series were done to obtain a standard curve for all mRNA samples. The threshold cycle was determined for target gene and endogenous control was used to calculate the relative expression of mRNA based on respective standard curve equation. Finally the expression of the target gene was normalized to the endogenous control gene.

WESTERN BLOT (Paper II-III)

Tissue Processing: Portion of the heart tissue (15-20 mg) were pulverized in micro centrifuge tubes over liquid nitrogen. Powdered heart tissue was homogenized by motor pestle in 400 µl ice cold lysis buffer containing 20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 % Triton X-100, 10 % (w/v) glycerol, 10 mM NaF, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM Na-pyrophosphate, 0.5 mM Na_3PO_4, 1 µg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 µg/ml aprotinin, 1 mM dithiothreitol (DTT) and 1 mM benzamidine. Homogenates were solubilized by rotation for 30 minutes at 4° C and subjected to centrifugation (12 000 x g for 15 minutes at 4° C). Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Western Blot Analysis: Protein phosphorylation and expression of Akt, p38 MAPK and GSK3-α/β were determined. An aliquot of tissue lysate (40 µg protein) was mixed in Laemmli sample buffer containing β-mercaptoethanol. Proteins were separated by 10% SDS-PAGE, transferred to polyvinylivenediflouride membranes (Millipore, Bedford, MA), and blocked in 5% non-fat dried milk in Tris-buffered saline with 0.02% Tween (TBST) for 2 h at room temperature. Membranes were incubated overnight at 4° C with phospho-specific antibodies for either Akt (Ser^{473}), p38 MAPK (1:1000, Cell Signaling Technology, Beverly, MA) and GSK3-α/β (1:1000, Upstate Biotechnology, Lake Placid, NY), washed in TBST, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:25000, Bio-Rad, Hercules, CA) for 1 hour at room temperature, followed by additional washing. Proteins were visualized by enhanced chemiluminescence's (ECL plus;

Amersham, Arlington Heights, IL) and quantified using densitometry and Molecular Analyst Software (Bio-Rad, Richmond, CA). After immunoblotting with phospho-specific antibodies, membranes were stripped as described (59) and immunoblotted for total Akt, p38 MAPK (1:1000, Cell Signaling Technology, Beverly, MA) and GSK3-α/β proteins (1:1000, Upstate Biotechnology, Lake Placid, NY) .

STATISTICAL ANALYSIS

Experimental design was done in a blinded manner including capillary and arteriolar analysis. All mRNA and Western blot experiments were repeated for more than two times in all experiments. Data are expressed as mean ± SEM. Student's Unpaired t- Test was used to reject the null hypothesis (Paper I). Coefficient of variation (CV %) for a single determination was calculated as described (60). Analysis of variance (ANOVA) was used for rejection of the null hypothesis for multiple group comparisons. If statistical nonhomogeneity was present, localization of group differences was done with Fisher's PLSD test (Paper II, III and IV). Non-parametric Mann-Whitney Rank Sum Test was used for Western blot data (Paper III). A P value of <0.05 was considered significant.

RESULTS

Paper I

Aims

The aim of this study was to explore the angiogenic effects of administration of a single (VEGF-A or Ang-1) growth factor and their combination (VEGF-A+Ang-1) in normal and induced acute myocardial infarcted heart.

Results

Highest protein expression of phVEGF-A_{165} in mouse hearts occurred between 24 and 48 hours (106± 31, 111± 28 and 41± 23 pg/ml after 24, 48 and 96 hours, respectively). No hVEGF-A expression was detected in saline injected hearts.

In the normal mouse heart, a single intramyocardial injection of either phVEGF- A_{165} or phAng-1 induced enhanced capillarization 10 days after injection by 16% (P< 0.005) and 13% (P<0.01), respectively (Fig. 9) : the combination had no additional effect on angiogenesis.

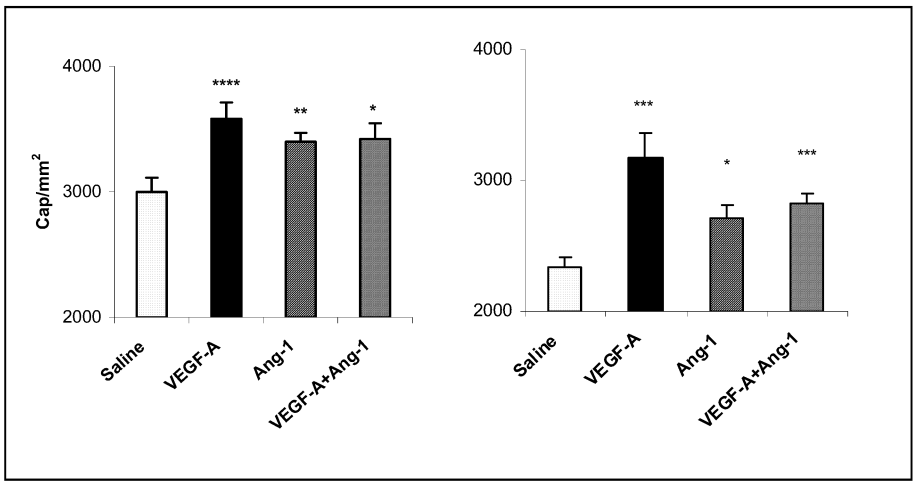


Figure 9: Capillary density in normal heart (left) and in AMI heart (right). * P<0.02, ** P< 0.01, ***P<0.003 and **** P<0.005 compared to saline treated heart.

In ischemic post infarction peri-infarct myocardium capillary density was reduced and phVEGF- A_{165} increased capillary density by 36 % ($P<0.003$) compared to saline (Fig. 9). phAng-1 and its combination with phVEGF-A_{165} also enhanced capillarization compared to control but not above the level observed by phVEGF-A_{165} alone.

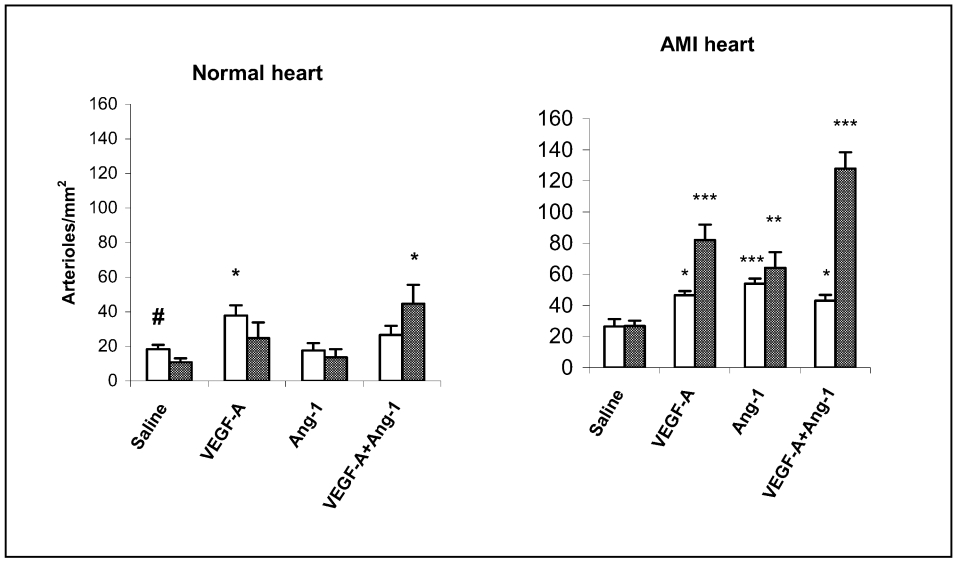


Figure 10: Density of large (open bar ≥ 20 µm diameter) and small (closed bar, ≤ 20 µm diameter) arteriolar density in normal heart (left) and AMI heart (right). # $P<0.03$ large compared to small in saline treated heart. * $P<0.02$, ** $P<0.01$ and $P< 0.001$ compared to saline.

In the normal heart the arteriolar density was higher after treatment with phVEGF-A_{165} ($P<0.002$) and phVEGF-A_{165} + phAng-1 ($P<0.02$) while phAng-1 alone did not change the density compared to saline (Fig.10). The arteriolar density after saline injection in the post-infarction myocardium compared to the non-ischemic normal heart was increased expressed both as density ($P<0.02$) and the ratio arteriolar to capillary density ($P<0.002$) (Fig.11). Interestingly and contrary to the normal heart, in the ischemic myocardium phAng-1 alone increased ($P<0.003$) arteriolar density similar to phVEGF-A_{165} alone. Further, the combination of factors had an additive effect with more than 6-fold

(P<0.001) and 3-fold (P<0.03) increase in ratio over saline-treated ischemic post-infarction myocardium and non-ischemic normal heart, respectively.

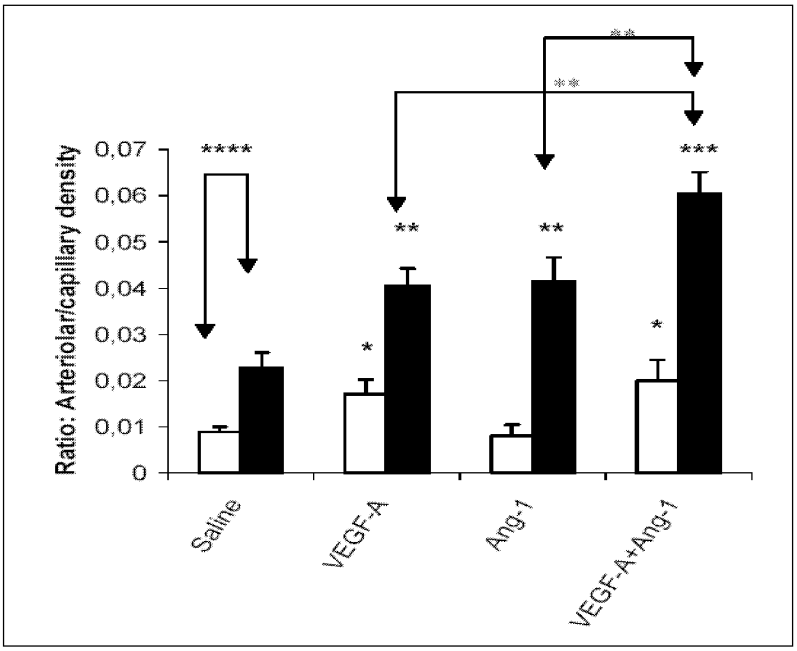


Figure 11: Ratio of arteriolar to capillary density in normal (open bar) and AMI (closed bar) model. * P<0.03, ** P<0.02, *** P< 0.001 and **** P< 0.002 compared to saline in the respective model if not otherwise indicated by arrows.

Conclusions

Overexpression of VEGF-A_{165} boosts an endogenous arteriogenesis in addition to a capillary angiogenesis in the ischemic post-infarction myocardium. Ang-1 in combination with VEGF-A further boosts this effect at the arteriolar level.

Paper II

Aims

To characterize the mechanism of the therapeutic angiogenic VEGF-angiopoietin booster effect on the basis of adaptations of the endogenous VEGF-angiopoietin system.

Results

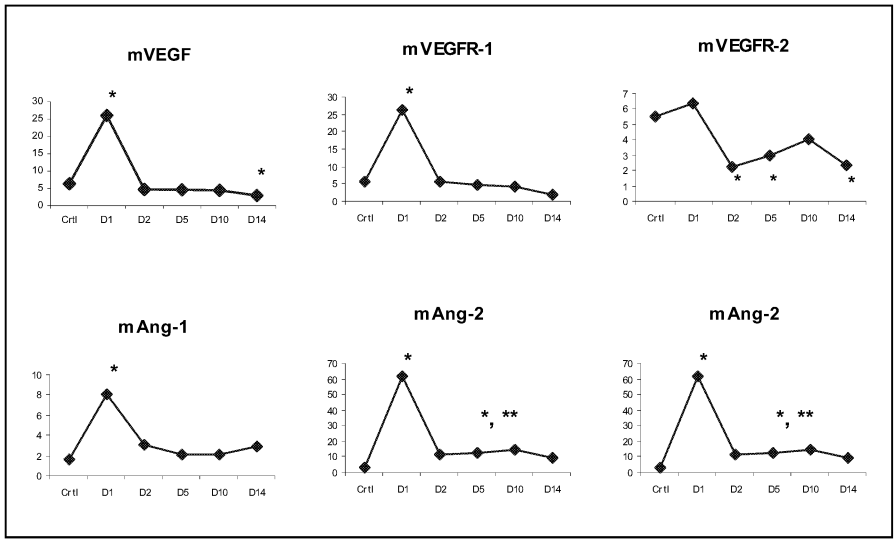


Figure 12: Above: mVEGF, VEGFR-1 and VEGFR-2 mRNA expression after AMI. ANOVA $P<0.001$, * $P<0.02$ compared to sham operated control. Below: mAng-1, mAng-2 and Tie-2 mRNA expression after AMI. ANOVA $P<0.02$ for mAng-1 and for mAng-2 and Tie-2 $P<0.001$, * $P<0.02$ compared to sham control. ** $P<0.02$ compared to day 1(D1). Values are mean ± SEM.

Peri-infarct myocardium at Day-1 (D1) post-infarction displayed that, the VEGF-angiopoetin system was upregulated except for VEGFR-2, which was unchanged (Fig.12). At D-2 VEGF-A, VEGFR-1, and Ang-1 expression were not different compared to the control. Ang-2 also decreased 3.7-fold but remained higher than the control. In the subacute phase (D5-D14), VEGFR-2 and Tie-2 were downregulated and Ang-1 was upregulated. Concurrently, phosphorylation of Akt and its downstream target GSK3-α/β was lower in the AMI heart suggesting an activated apoptosis process during the acute ischemia.

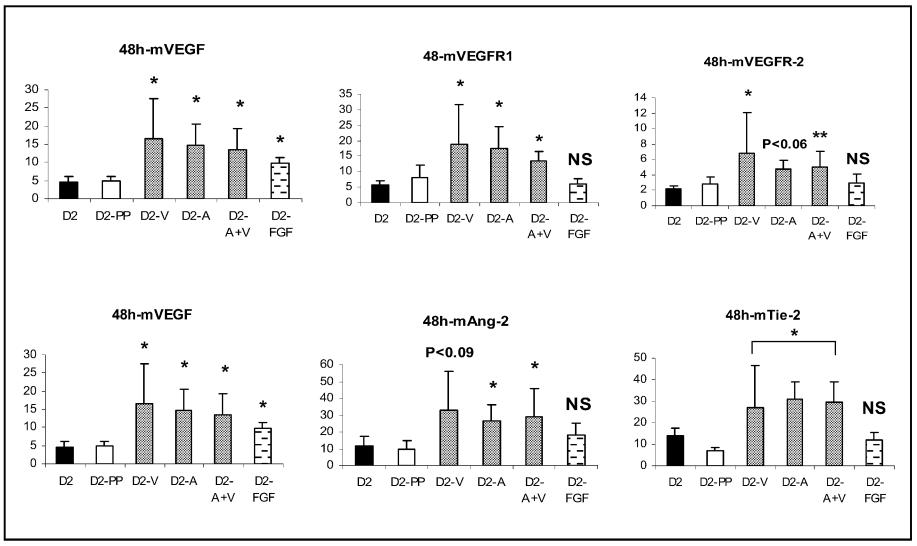


Figure 13: Modulation of the VEGF / angiopoietin system at 48 hours (D2) after AMI by placebo plasmid (PP), phVEGF-A_{165} (V), phAng-1 (A) and combination (V+A). Above: mVEGF, mVEGFR-1 and mVEGFR-2. ANOVA P<0.001, * P<0.05 compared to PP control (D2-PP). Below: mAng-1, mAng-2 and mTie-2. ANOVA P<0.02 for mAng-1 and mAng-2, P<0.001 for mTie-2, * P<0.05 compared to PP control (D2-PP). Values are mean ± SEM.

At D-2 overexpressed growth factors (V, A, and V+A) stimulated the mouse endogenous VEGF-Ang system while the placebo plasmid had no such effect (Fig.13). This stimulatory effect was specific to VEGF and angiopoietin overexpression since FGF overexpression essentially did not stimulate the VEGF-angiopoietin system. The stimulation of the VEGF-Angiopoetin system was independent of Akt and GSK phosphorylation since no differences of Akt and GSK3 phosphorylation were detected in the treatment groups.

Conclusions

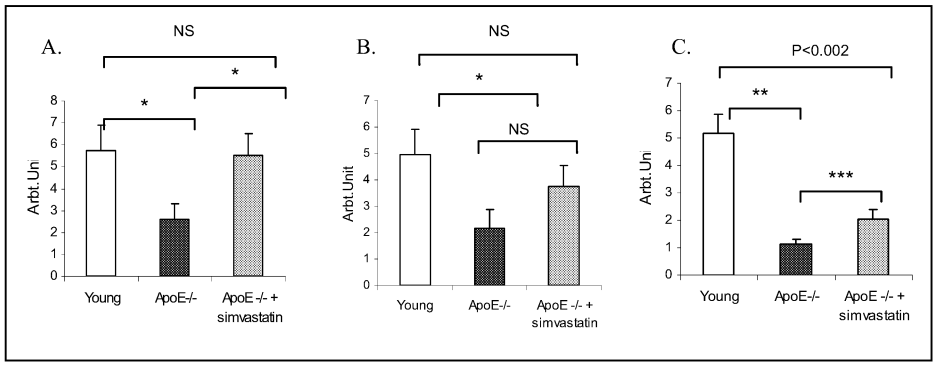
Therapeutic overexpression of VEGF-A_{165} and angiopoietin-1 both upregulate the endogenous ligands / receptors of the VEGF-angiopoietin system as mechanisms of higher arteriolar density, a condition independent of Akt and GSK3 protein phosphorylation during acute myocardial ischemia.

Paper III

Aims

To investigate the effects of myocardial VEGF gene transfer with or without simvastatin on vascular growth response in ApoE$^{-/-}$ knockout mice and if additive simvastatin-related effects on capillary growth were associated with effects on the endogenous VEGF system including both the ligand and its receptors.

Results



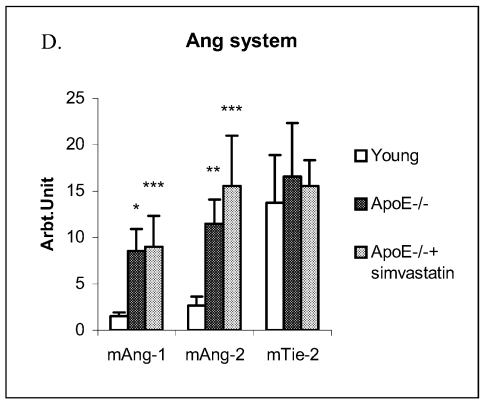


Figure 14: mRNA expression in young and ApoE $^{-/-}$ mice without and with simvastatin. (A) mVEGF, (B) VEGFR-1 (C) VEGFR-2 and (D) mAng-1, mAng-2 and Tie-2 mRNA expression. Values are mean ± SEM.

Compared with normal mice, one-year-old ApoE$^{-/-}$ mice fed on a high-fat diet had about 30% less myocardial capillary ($P < 0.001$) and arteriolar ($P < 0.03$) densities, associated

with decreased VEGF (55%), VEGFR-1 (56%) and VEGFR-2 (78%) mRNA expressions (Fig.14) and myocardial endothelial nitric oxide synthase (eNOS) production (58%). By contrast, Ang-1 (five-fold, $P < 0.02$) and Ang-2 (four-fold, $P < 0.01$) mRNA expressions were increased in the ApoE$^{-/-}$ hearts compared to young control (Fig.14D). No change was observed in Tie-2 gene expression.

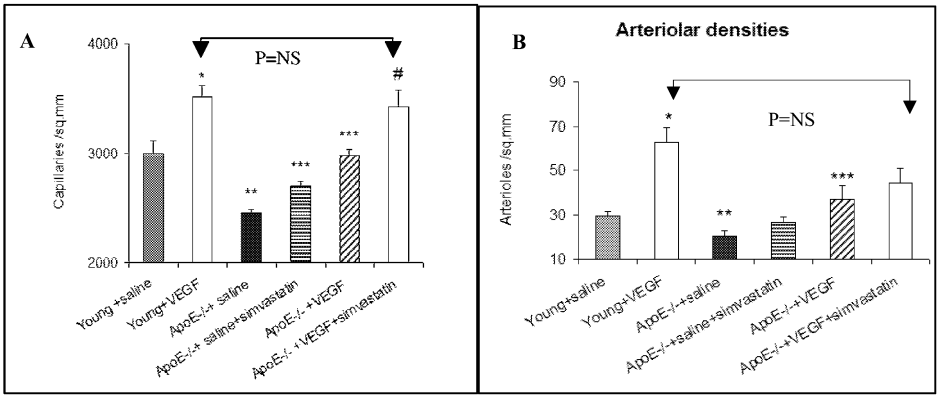


Figure 15: (A) Capillary densities in the young control and in the ApoE $^{-/-}$ mice, thoracotomy with saline or VEGF-A gene transfer without or with simvastatin treatment. * $P < 0.01$ young + saline vs. young + VEGF, ** $P < 0.002$ young saline vs. ApoE $^{-/-}$ + saline, *** $P < 0.0001$, ApoE $^{-/-}$ + saline vs. ApoE $^{-/-}$ saline + simvastatin and ApoE $^{-/-}$ + saline vs. ApoE $^{-/-}$ + VEGF and # $P < 0.03$ ApoE $^{-/-}$ + VEGF vs. ApoE $^{-/-}$ + VEGF + simvastatin. (B) Arteriolar densities in young mice and in ApoE $^{-/-}$ mice as above. * $P < 0.001$ young + saline vs. young + VEGF, ** $P < 0.03$ young + saline vs. ApoE $^{-/-}$ + saline, and *** $P < 0.04$ ApoE $^{-/-}$ + saline vs. ApoE $^{-/-}$ + VEGF. Values are mean ± SEM.

Phosphorylation of antiapoptotic Akt was lower and proapoptotic p38 mitogen-activated protein kinase (MAPK) was higher in the ApoE$^{-/-}$ mice compared with control young mice. Intramyocardial VEGF gene transfer increased capillary and arteriolar densities in the ApoE$^{-/-}$ mice, and simvastatin treatment further enhanced capillary density ($P < 0.03$) to a level similar to that of normal mice (Fig.15).

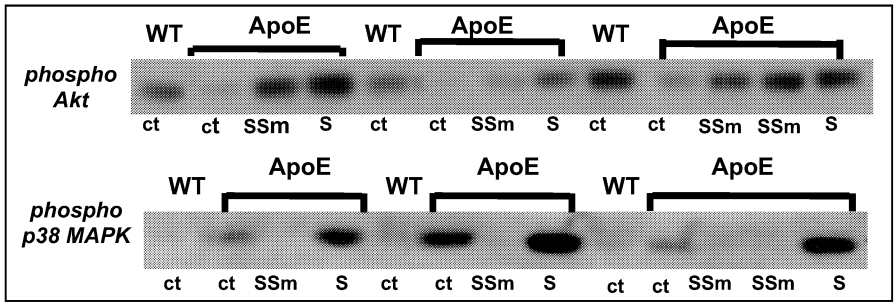


Figure 16: Western blot detecting Akt and p38 MAP kinase phosphorylation along with total Akt as loading control. Young control and ApoE$^{-/-}$ with simvastatin treatment after thoracotomy and intramyocardial saline injection (10µl) (N=3, in each group). ct: control, SSm: saline + simvastatin, S: saline, WT: young control, ApoE: ApoE$^{-/-}$.

Simvastatin did not change the lipid profile but blocked p38 MAPK phosphorylation in the ApoE$^{-/-}$ myocardium (Fig.16). Concurrent with these changes, there were increased levels of expression of mVEGF ($P < 0.04$) and VEGFR-2 ($P < 0.03$) mRNAs and increased production of eNOS ($P < 0.05$) in the ApoE$^{-/-}$ mice while no changes were detected in the angiopoietin system.

Conclusions

Myocardial angiogenesis in the aged and hypercholesterolemia ApoE$^{-/-}$ mouse is impaired which is associated with down-regulation of endogenous VEGF and its receptors, specifically the VEGFR-2. Interestingly, ang-1 and ang-2 were upregulated. The increased myocardial angiogenesis in the ApoE$^{-/-}$ mice following transient overexpression of VEGF is further increased by additional simvastatin treatment which occurs concurrently with the stimulation of the VEGF system, increased eNOS production and reduction in p38 MAPK phosphorylation.

Paper IV

Aim

To investigate effects of inhibition of the renin angiotensin system (RAS) on VEGF-A gene transfer induced myocardial angiogenesis.

Results

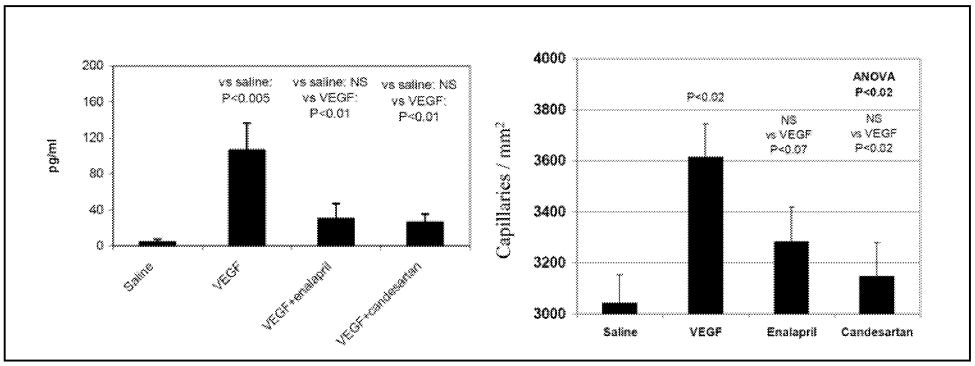


Figure 17: Left: hVEGF-A expression after 24 hours with enalapril or candesartan. * P< 0.02 and ** P< 0.05 compared to phVEGF-A_{165} alone. Right: Capillary density after 10 days from a single intramyocardial injection of either placebo plasmid or phVEGF-A_{165} with or without enalapril or candesartan. * *P*<0.001 phVEGF-A_{165} vs. placebo plasmid, ** *P*<0.03 phVEGF-A_{165} + enalapril vs. phVEGF-A_{165} alone and *** *P*<0.01 phVEGF-A_{165} + candesartan vs. phVEGF-A_{165} alone. Values are mean ± SEM

phVEGF-A_{165} increased myocardial capillary density associated with induced expression of VEGF-A (Fig.17). Increased myocardial capillary density was counteracted by both enalapril (P<0.07) and candesartan (P<0.02) and then did not differ from saline injected control. phVEGF-A_{165} induced myocardial hVEGF-A expression (110±15 pg/heart, P<0.0001) and enalapril and candesartan decreased (P<0.01) expression of hVEGF-A to a level similar to the control. phVEGF-A_{165} overexpression in normal heart upregulated (P<0.0001) endogenous mVEGFR-2 receptor activation. Treatment of candesartan downregulated endogenous mVEGF-A (P<0.0001) and mVEGFR-2 (P<0.0001)

expression (Fig.18) compared to normal myocardium. Enalapril or candesartan did not effect luciferase expression.

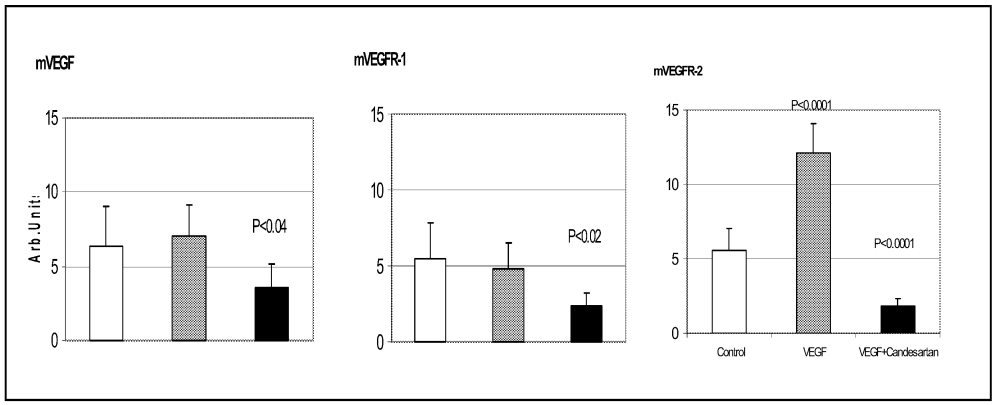


Figure 18: mRNA expression of mVEGF-A, mVEGFR-1 and mVEGFR-2 24 hours after sham operated control or myocardial injection of phVEGF-A165 with or without candesartan treatment. Values are given as mean with SEM. ANOVA showed statistical inhomogeneity for all three specimens. P values refer to group differences against baseline.

Conclusions

Enalapril and candesartan both specifically inhibit phVEGF-A_{165} induced myocardial angiogenesis in the noncompromised heart. The mechanism of inhibition is a combination of inhibition of cardiac hVEGF-A expression and of decreased endogenous expression of the mVEGF ligand / receptor system.

GENERAL DISCUSSION

Angiogenic gene therapy for therapeutic myocardial angiogenesis may provide additional treatment options for ischemic heart disease when conventional treatments are not capable to improve symptoms of ischemia, myocardial function and quality of life. Myocardial gene therapy was tested with different gene transfer protocols and angiogenic response was evaluated with different techniques (3, 30, 31). The angiogenic response can be different between different tissues depending on mode of delivery, local tissue environment and demands as well as duration of expression of the gene therapy. Advancement of the knowledge of the molecular basis of vessel growth in IHD will help to design an optimum therapeutic strategy and treatment.

Combination Therapy*:*

VEGF-A as a sole angiogenic factor was selected in many of the clinical studies due to its high specificity to endothelial cells and its angiogenic response during ischemia (3, 5, 24). A dense capillary network is critical for cardiomyocytes for the transportation of oxygen and nutrients since vascular network comprises 35% of the volume of the heart (61). For a mature vascular network some of the newly formed capillaries are in need of pericytes and smooth muscle cells. Transient overexpression of VEGF-A induces capillaries and most unexpectedly also arterioles and especially on small arterioles (Paper I). Thus the net angiogenic effect from overexpressing VEGF-A seems to be greater in the ischemic myocardium than in the normal heart suggesting additional adaptation of the endogenous angiogenic system during myocardial ischemia. Ang-1 overexpression also induces an increase in capillary density. Interestingly arteriolar density increased only in

the ischemic post-infarction myocardium. This paradigm of angiogenic response of Ang-1 can be explained from a recent study where it has been shown that angiogenic activity of Ang-1 largely depends on endogenous VEGF expression (62). Interestingly our data also provide further mechanistic evidence, that Ang-1 overexpression stimulates the endogenous VEGF system including its receptors VEGFR-1 and VEGFR-2 and also stimulates the endogenous angiopoietin system (Paper II). Ischemic episode is associated with upraise of VEGF and its receptors expression (63-67). However, in agreement with previous studies we did observe an early upregulation of the VEGF and angiopoetin system during acute ischemia. In the chronic phase of ischemia, expression of the VEGF and angiopoetin system is equal to normal myocardium except for VEGFR-2 which decreases below the normal myocardial level. The mechanism of downregulation of the VEGF-Angiopoetin system in the late chronic phase is unknown. At the physiological level, it could reflect a hibernating cardiomyocyte phenotype where the myocardium adapts to available blood supply with less contractility and vascular capacity. A similar adaptation was reported in the human ischemic myocardium where the VEGF system was similar to non-ischemic myocardium (68); however at the molecular level, phosphorylation of Akt might be one of the important factors considering its role in the angiogenic process (69,70). Indeed, our data also provide evidence that in the subacute chronic phase the phosphorylation of Akt including its downstream effectors GSK-3 α / β is also lower (Paper II), suggesting increased apoptosis and negative effect on the angiogenic process. However, at our experimental time point we were unable to show any effect on the Akt system following overexpression of the growth factors but it dose not exclude such a possibility.

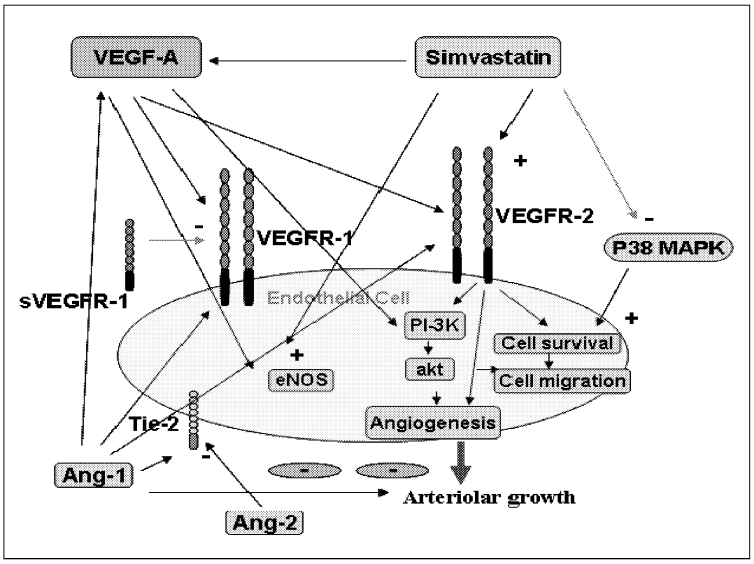


Figure 19: Mechanism of VEGF-A / Ang-1 mediated angiogenic effect (Paper I, II) and interaction of simvastatin with VEGF-A gene transfer for angiogenesis (Paper III).

In fact our data showed that arteriolar density increased more than 60-fold over the increase in capillary density during ischemia (Paper I). Theses results are consistent with observations where combination of VEGF and Ang-1 enhanced perfusion; decreased leakiness associated with increased vascular growth (71), enhanced collateral development and increased capillary growth (72). The observations that transient overexpression of VEGF-A and Ang-1 stimulated upregulation of their endogenous ligand receptor systems suggest the mechanism for the pronounced angiogenic effect in the ischemic myocardium (Fig.19).

Gene Therapy in Combination with Drug Treatment:

Complex and advanced myocardial ischemia is often associated with systemic or metabolic disorders such as hypertension and hyperlipidaemia with highest prevalence in

the elderly population. Because of the potential for therapeutic angiogenesis in such conditions, we investigated effects of myocardial gene therapy along with drug treatments that are frequently used in these patients. Aging and hypercholesterolemia in the ApoE$^{-/-}$ mouse heart were associated with less capillary and arteriolar densities and lower expression of endogenous VEGF and its receptors and of eNOS. Interestingly the expression of the VEGFR-2 receptor and the phosphorylation of Akt were markedly low in the myocardium of the ApoE$^{-/-}$ heart at baseline. VEGF-A gene transfer combined with simvastatin treatment normalized the vascular densities. Thus it appears that the impaired angiogenic state in the aged hypercholesterolemia ApoE$^{-/-}$ mouse can be reversed by VEGF-A overexpression combined with simvastatin that in its turn upregulates VEGFR-2 and eNOS as major factors in the angiogenic process.

The enhanced angiogenic effect with the combination treatment seems to be related to simvastatin mediated non-lipid related divergent effects as shown in the present work as well as reported by others (50-53). p38 MAPK belongs to a family of stress-activated serine/threonine protein kinases and induces apoptosis caused by ischemia in a variety of cell types including endothelia, smooth muscle and cardiomyocytes (73, 74). Importantly, simvastatin inhibited the proapoptotic protein p38 MAPK phosphorylation and also stimulated recruitment of endothelial progenitor cells (75) suggesting additional angiogenic mechanisms in our model. The study presents a new aspect of therapeutic myocardial gene therapy in connection with simvastatin in a setting of hypercholesterolemia and aging. Accordingly, our data suggest that, impaired myocardial angiogenesis during hypercholesterolemia and aging can be reversed by transient overexpression of VEGF-A in combination with simvastatin treatment (Fig.19).

The renin angiotensin system (RAS) is essential for regulating vascular homeostasis, blood pressure and left ventricular hypertrophy. Evidence suggests that RAS during myocardial failure is activated both locally in the heart and systemically resulting in vasoconstriction and secondary antiangiogenic effects (Fig.20) (77). Our data showed that RAS inhibition counteracted phVEGF-A gene transfer mediated angiogenesis. This effect was associated with significantly reduced plasmid VEGF-A protein expression. As there was no significant difference in luciferase protein expression, it is suggested that RAS inhibition is not associated with effects on cellular or nuclear uptake of plasmid or on inhibition of the CMV promoter. Rather it may be associated with decreased transcription-translation, VEGF protein stability or increased elimination. Also AT_1 receptor (AT_1-R) blockade downregulated the cardiac endogenous VEGF ligand / receptor system. Also RAS inhibition down regulated endogenous mRNA expression of VEGF, VEGFR-1 and VEGFR-2. This effect might depend on RAS system which is

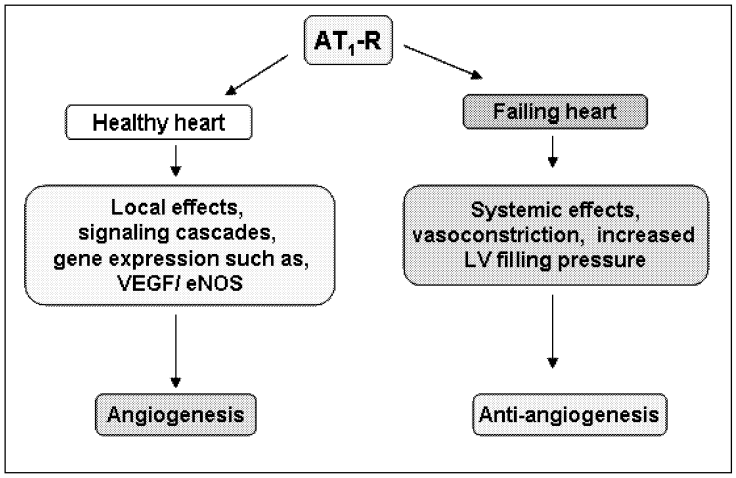


Figure 20: **AT_1- receptor (AT_1-R) activation and its effects in the healthy and the failing heart:** In the healthy heart by primarily local activity it stimulates angiogenesis involving local signaling cascades and gene expression including VEGF and eNOS. In the failing heart primarily by systemic effects it counteracts angiogenesis. Inhibition of AT_1-R counteracts angiogenesis in the healthy heart while stimulates angiogenesis in the failing heart.

involved in diverse cellular signaling cascades, like stimulating MAP kinase, tyrosine kinases as well as various transcription factors (Fig.20) (52). Anti angiogenesis following RAS inhibition has previously been reported in hypoxia (77), in different organs such as: heart (78), skeletal muscle (79) as well in diabetic retinopathy (80). RAS inhibition has also been reported to reduce tumor growth, which provides further evidence of its anti-angiogenic role (81, 82). Interestingly, the balance between AT1 and AT2 receptor expression may explain the anti-angiogenic effect observed by RAS inhibition particularly by AT1 receptor blockade. AT1 receptor stimulation promotes angiogenesis and vasoconstriction and AT2 receptor stimulation counteracts angiogenesis that may be due to apoptosis of endothelial and smooth muscle cells (83, 84) and induction of pro-apoptotic protein Bax (85). These results emphasize the complexity of RAS actions as well as diverse actions on different tissue systems. Thus our data are in keeping with previous findings (78, 86-88) and suggest that RAS inhibition counteracts VEGF-A mediated angiogenesis both at the VEGF-A expression level as well as at the myocardial cellular level where it inhibits particularly expression of the endogenous VEGF system.

GENERAL CONCLUSIONS

1. Transient overexpression of combination of VEGF and angiopoietin has an angiogenic booster effect.
2. Overexpresion of VEGF-A and Ang-1 stimulate the endogenous VEGF and angiopoietin ligand receptor system.
3. Aging and hypercholesterolemia have negative effects on angiogenesis, VEGF-A gene transfer and simvastatin treatment corrected these negative effects.
4. VEGF-A gene transfer concurrent RAS inhibition induced an inhibitory effect on angiogenesis in the non-compromised heart.
5. Commonly used cardiovascular drugs can stimulate or inhibit angiogenesis.

Future Perspective

A better understanding of myocardial ischemic events at the cellular and molecular levels is a key to increased therapeutic efficacy of myocardial angiogenesis. Such therapeutic interventions may need more than a single growth factor with sustained expression and perhaps with combination of transcription factor overexpression or with an anti-apoptotic strategy.

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